



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/82, 15/55, 1/21, 1/18, 5/10,</b> <b>A01H 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/24448</b> <b>(43) International Publication Date:</b> 10 July 1997 (10.07.97)
<b>(21) International Application Number:</b> PCT/GB96/03239 <b>(22) International Filing Date:</b> 24 December 1996 (24.12.96) <b>(30) Priority Data:</b> 9526613.6 28 December 1995 (28.12.95) GB <b>(71) Applicant (for all designated States except US):</b> NICKERSON BIOCEM LIMITED [GB/GB]; Cambridge Science Park, Milton Road, Cambridge CB4 4GZ (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TAYLOR, Mark, Andrew [GB/GB]; 9 Newhall Gardens, Dundee DD2 1TW (GB). DAVIES, Howard, Vivian [GB/GB]; 2 Station Road, Inver- gowrie, Dundee DD2 5DU (GB). <b>(74) Agents:</b> CORNISH, Kristina, Victoria et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> POTATO ALPHA-GLUCOSIDASE GENE  <b>(57) Abstract</b>  The present invention provides recombinant or isolated nucleic acid encoding an $\alpha$ -glucosidase enzyme, especially those nucleic acid sequences encoding a plant $\alpha$ -glucosidase enzyme. Antisense nucleic acid is also provided, as well as the use of both the isolated or recombinant sequences and the antisense sequences. Uses of the invention include enhancing and reducing expression of $\alpha$ -glucosidases and the provision of novel starches.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## POTATO ALPHA-GLUCOSIDASE GENE

This invention relates to the fields of plant biotechnology and plant genetic engineering. In particular, it relates to the production of novel starches by plants and to the manipulation of starch-sugar conversions in transgenic plants by changing the cellular activity of the  $\alpha$ -glucosidase enzyme. More specifically, it relates to nucleic acid sequences encoding  $\alpha$ -glucosidase enzymes and the potential use of such sequences when expressed in plants.

It is known that the mobilisation of starch by hydrolysis in plants involves the enzymes  $\alpha$ -amylases,  $\beta$ -amylase, debranching enzyme and  $\alpha$ -glucosidase. However, the precise roles of the of starch degrading enzymes and their mechanisms of action remain unclear. One view is that  $\alpha$ -amylases are the only enzymes that use native starch as a substrate for hydrolysis. Accordingly, the role of  $\beta$ -amylase, debranching enzyme and  $\alpha$ -glucosidase is to hydrolyse the dextrans released by  $\alpha$ -amylase activity on the granule (reviewed by Beck and Zeigler, 1989). Conversely, some evidence suggests that  $\alpha$ -amylase cannot act on starch grains alone and requires other factors for starch degradation (Stamberg and Bailey, 1939). A good candidate for this  $\alpha$ -amylase activating factor is  $\alpha$ -glucosidase (Schwimmer, 1945). In fact some  $\alpha$ -glucosidases have been shown to catalyse the hydrolysis of soluble starch, although at very low rates (Yamasaki and Konno, 1985). In germinating barley there was a high degree of synergistic starch hydrolysis when  $\alpha$ -amylase and  $\alpha$ -glucosidase were incubated with starch granules (Sun and Henson, 1990). More recently, it has been demonstrated that a pea chloroplastic  $\alpha$ -glucosidase is capable of initiating chloroplastic starch grain degradation (Sun et al., 1995). This may cause starch grains to be more susceptible to further hydrolysis by

other enzymes. Thus, it appears that the importance of  $\alpha$ -glucosidases in starch hydrolysis may have been underestimated.

- 5       $\alpha$ -Glucosidases catalyse the hydrolysis or transfer of the terminal  $\alpha$ -D-glucosyl residues of  $\alpha$ -D-glucosidically linked carbohydrates. A general feature is that, unlike other starch degrading enzymes, a broad range of compounds are substrates for these enzymes.
- 10      Generally, as well as catalysing the hydrolysis of maltose, they will also use oligosaccharides (maltodextrins) and polysaccharides (amylose, amylopectin and glycogen) as substrates (Yoshikawa et al., 1994). Additionally, most  $\alpha$ -glucosidases will hydrolyse a range
- 15      of glucobioses containing  $\alpha$ -1,2,  $\alpha$ -1,3,  $\alpha$ -1,4 and  $\alpha$ -1,6 bonds. This may be particularly important if all these type of linkages are present in vivo, as other starch degrading enzymes such as  $\alpha$ -amylase cannot catalyse the breakdown of  $\alpha$ -1,2 and  $\alpha$ -1,3 bonds (Sun et
- 20      al., 1995). Some evidence suggests that these types of linkages do exist in starch grains (Abdel-Akher, M. et al., 1952, Wolfrom and Thompson, 1956).  $\alpha$ -Glucosidases also catalyse transglycosylation reactions that may be important in vivo. For example, isomaltose, maltotriose
- 25      and panose can be synthesised from maltose (Yamasaki and Suzuki, 1980, Yamasaki and Konno, 1985) and kojibiose, nigerose and maltose can be synthesised from soluble starch (Chiba, 1988).
- 30      It is well known that plants with novel characteristics can be produced by the expression of gene sequences introduced by transformation procedures such as *Agrobacterium*-mediated or vector mediated transformation methods or physical transformation methods such as

biolistics, chemical or electrical transfection or micro-injection, introducing genes or DNA sequences (Draper et al., 1988). Despite the widespread occurrence of  $\alpha$ -glucosidases in higher plants and their importance in starch and carbohydrate metabolism, no  $\alpha$ -glucosidase genes from plants have been characterised.

This has the disadvantage of preventing a transgenic approach for the manipulation of the expression of  $\alpha$ -glucosidase genes.

Accordingly, a first aspect of the present invention provides a recombinant or isolated nucleic acid encoding an  $\alpha$ -glucosidase enzyme, preferably a plant  $\alpha$ -glucosidase enzyme. Nucleic acid according to the present invention is preferably DNA but also includes cDNA and RNA. The  $\alpha$ -glucosidase enzyme of the present invention includes sequences which are preferably obtainable from plants or microbes including, in particular, the plants: potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam, amongst other species.

The present invention also includes, according to all aspects, mutations and fragments of nucleic acid sequences encoding  $\alpha$ -glucosidases (preferably  $\alpha$ -glucosidases obtainable from plants) and mutations and fragments of amino acid sequences of those  $\alpha$ -glucosidase enzymes. The present invention particularly includes nucleic acid and amino acid sequences of  $\alpha$ -glucosidase which are obtainable from the potato cultivars Record, Desiree, Binje or Russet Burbank as well as mutants and fragments thereof.

Fragments of nucleic acid according to the present

invention include 9 or more, preferably 12 or more, preferably 15 or more, preferably 18 or more bases and the corresponding number of amino acids. When used to describe the invention, the phrase 'a part of' means any size fragment thereof.

The recombinant or isolated nucleic acid according to the first aspect of the invention preferably encodes for an enzyme having the activity of a 105.4 kD  $\alpha$ -glucosidase enzyme of potato, especially the potato cultivar Record.

The recombinant or isolated nucleic acid according to the first aspect of the invention most preferably encodes at least part of (ie. a fragment of) the amino acid sequence as shown in Figure 2. The recombinant or isolated nucleic acid preferably encodes an amino acid sequence having more than 29% identity with the sequence shown in Figure 2, more preferably 40% identity, even more preferably 60% identity.

The recombinant or isolated nucleic acid may have a coding sequence operatively linked to a promoter. The promoter may be constitutive, for example the well known CaMV35S promoter or inducible, for example, the GAL/GAL10 promoter. The promoter may also be tissue-specific, for example, the tuber-specific promoter GBSSI, the tuber-specific patatin I promoter (Kim et al), the tomato fruit-specific E8 promoter (Lincoln et al) and the promoter of the small subunit of the ribulose-1,5-biphosphate carboxylase gene (Coruzzi et al).

According to a second aspect of the invention, there is provided a recombinant or isolated nucleic acid comprising a promoter which naturally drives expression

of a nucleic acid sequence encoding an  $\alpha$ -glucosidase enzyme, preferably a plant  $\alpha$ -glucosidase enzyme. The coding sequence for the  $\alpha$ -glucosidase is preferably as described above, according to the first aspect of the invention.

The recombinant or isolated nucleic acid according to the first and second aspects of the invention, when expressed, may result in enhanced starch and/or other carbohydrate breakdown, preferably in a plant. It may also result in novel starch structures. The enhanced breakdown is a result of increased expression of the  $\alpha$ -glucosidase enzyme, usually compared to endogenous levels. According to promoters used, for example, a tissue specific promoter, the increased levels of  $\alpha$ -glucosidase can be limited to desired tissues, such as plant storage organs (for example, potato tubers).

The recombinant or isolated nucleic acid of the first and second aspects of the invention may include a signal sequence in translational fusion with the  $\alpha$ -glucosidase coding sequence. Such a sequence may enable the expression of an introduced  $\alpha$ -glucosidase sequence specifically in a cellular organelle. For example, the GBSSI signal sequence targets expression to the plastid in potato tubers (Visser et al 1989).

According to a third aspect of the invention there is provided antisense nucleic acid to nucleic acid encoding  $\alpha$ -glucosidases of the present invention. Antisense nucleic acid is well understood in the art. Thus the present invention provides antisense nucleic acid which comprises a transcribable strand of nucleic acid, the transcribed nucleic acid being complementary to at



least part of a strand of nucleic acid which is transcribed from a nucleic acid sequence encoding a  $\alpha$ -glucosidase enzyme. Preferably, the nucleic acid sequence encoding the  $\alpha$ -glucosidase enzyme is obtainable from a plant, but it may also be obtainable from other sources, such as microbial sources (eg. yeast). The transcribable nucleic acid of the antisense and/or encoding the  $\alpha$ -glucosidase may be recombinant. The nucleic acid encoding the  $\alpha$ -glucosidase may be a 'natural' (endogenous) nucleic acid sequence (which may also be recombinant) or may be a modified sequence. The nucleic acid encoding the plant  $\alpha$ -glucosidase enzyme may be obtainable from a potato, in particular, the potato cultivar Record. The nucleic acid encoding the plant  $\alpha$ -glucosidase enzyme may preferably encode for an enzyme having the activity of a 105.4 kD  $\alpha$ -glucosidase enzyme of potato.

The antisense nucleic acid is advantageously complementary to part of a nucleic acid sequence encoding for the amino acid sequence shown in Figure 2. Preferably the antisense nucleic acid is complementary in the same level of identity as described hereinbefore for the recombinant or isolated nucleic acid. In this way, the transcribable strand of nucleic acid from the antisense nucleic acid will be complementary to at least part of the strand of the nucleic acid which is transcribed from the nucleic acid encoding a plant  $\alpha$ -glucosidase enzyme.

The antisense nucleic acid may be operatively linked to a promoter, as described according to the first aspect of the invention.



According to a fourth aspect of the invention there is provided nucleic acid capable of disrupting the proper expression of an  $\alpha$ -glucosidase gene, preferably a plant  $\alpha$ -glucosidase gene, most preferably an  $\alpha$ -glucosidase gene of potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam. The disruption may be by any recombinant nucleic acid technology, including those well known in the art. The nucleic acid capable of the most effective disruption, disrupts proper expression of a gene encoding an  $\alpha$ -glucosidase enzyme having the activity of a 105.4 kD  $\alpha$ -glucosidase enzyme of potato. Nucleic acid capable of disrupting the proper expression of an  $\alpha$ -glucosidase gene, according to the invention, may comprise nucleic acid obtainable from a source other than a plant eg. a microbial source, in particular a yeast.

The nucleic acid according to any aspect of the invention may comprise a 5' transcription regulation sequence such as the 5' transcription regulation sequence derived from the Cauliflower Mosaic Virus 35S gene, GBSSI, patatin, E8 and the promoter of the RUBISCO small subunit gene.

Nucleic acid, according to any aspect of the invention may comprise part of a vector. Suitable vectors are well known in the art and include cloning vectors such as lambda Zap II (Stratagene) and expression vectors such as pYES2 (Invitrogen). Preferably, the vector comprises one or more selectable markers such as antibiotic resistance.

A construct, comprising nucleic acid according to any one of the aspects of the invention, and including the vectors according to the invention can be introduced into a host cell by transfection or transformation. Such methods are well known in the art. The host cell can

than be used to express and monitor expression of  $\alpha$ -glucosidase activity or can be used to clone plants, microbes and/or parts of plants, including tissue culture.

5

Preferably, a construct comprising a promoter operatively linked to a nucleic acid sequence, encoding an  $\alpha$ -glucosidase enzyme can be transformed into a plant or other organism, such as a microbe, eg. yeast. Suitable plants include potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam amongst others. Suitable transformation techniques are well known in the art as described above. The transformation results in plants, or other organisms, at least some of the cells of which contain a foreign chimeric nucleic acid sequence composed of a promoter operatively linked to nucleic acid encoding an  $\alpha$ -glucosidase enzyme. These constructs can include nucleic acid encoding any  $\alpha$ -glucosidase (eg. plant, or microbial) for introduction into plant, microbial or animal cells.

20

In this way, the nucleic acid of the present invention can be used to modify starch and/or other carbohydrate breakdown in material, in particular in plant material. Clearly, the most effective use of such systems are when the promoter sequence drives preferential expression of the nucleic acid in plant storage organs and/or seeds. As a result, the rate of starch and/or other carbohydrate breakdown in plant materials can be modified as well as the potential to produce novel starches and/or other carbohydrates in plants, preferably plant storage organs.

25

30

The nucleic acid sequences, constructs, vectors etc., according to the first aspect of the invention, can be

used to obtain enhanced  $\alpha$ -glucosidase activity in plant or other organisms material/tissues. A consequence of this is a more efficient breakdown of starch and/or other carbohydrates and the opportunity to product novel starches and/or other carbohydrates. This is a benefit to processes which lead to the production of glucose and/or other starch derived products such as maltose and maltose oligosaccarharides. These includes the malting and brewing process in which the efficiency of starch conversion to glucose will be increased, leading to increased spirit yield. Additionally, increased starch breakdown will increase the glucose content of plant tissues and will consequently enhance the flavour characteristics of plant tissues (for example the fruit of tomato, strawberry, raspberry, blackcurrent).

Furthermore, the nucleic acid sequences, constructs, vectors, etc., according to the third and fourth aspects of the invention can be used to obtain reduced  $\alpha$ -glucosidase activity in plant or microbial material. Inhibition of  $\alpha$ -glucosidase activity by transformation of plant tissue with constructs containing the potato  $\alpha$ -glucosidase sequence in antisense orientation will result in inhibition of starch breakdown in seeds and other storage organs. This will be of use in developing tissues, in storage tissue and in germinating systems. Applications also include inhibition of pre-sprouting in cereals, sprout control in potato, minimising starch and dry weight losses in storage and during cooking or processing. A potential outcome is restricted starch turnover rates and a subsequent increase in the amount of starch deposited.

The nucleic acid sequences of the present invention can

be used as hybridisation probes to clone  $\alpha$ -glucosidase genes from other sources and species, for example microbes and pea, maize, wheat, rice, barley, sweet potato, cassava and yam (respectively).

5

A further aspect of the present invention also provides for novel starch structures, in particular in plant materials. The novel starches are a result of the  $\alpha$ -glucosidase activity at differing levels and/or from differing sources. The novel starch structures may have a changed branched structure and/or a changed branch length.

10

The invention is illustrated by the accompanying drawings in which:

15

Figure 1. shows the nucleotide sequence of the coding strand of the potato cultivar Record cDNA that encodes  $\alpha$ -glucosidase.

20

Figure 2. shows the deduced amino acid sequence from the cDNA sequence in Figure 1.

Figure 3. shows a comparison of the deduced amino acid sequence of the potato  $\alpha$ -glucosidase (labelled pot) with that of the human lysosomal sequence (labelled hum). The comparison was carried out using the default parameters of the GAP programme of the GCG package.

25

Figure 4. shows growth of yeast strains. (o) non-transformed ABYSMAL81 with 2% glucose as carbon source, (●) ABYSMAL81 transformed with pMAL1YES2 with 0.1% galactose and 2% maltose as carbon source, (□) ABYSMAL81 with 0.1% galactose and 2% maltose as carbon source and

30

(■) ABYSMAL81 transformed with pYES2 with 0.1% galactose and 2% maltose as the carbon source.

5 Figure 5. shows  $\alpha$ -glucosidase activity in crude extracts of ABYSMAL81 transformed with pMAL1YES2 measured in the pH range 3-9.

The invention is illustrated by the following non-limiting examples.

10

*Example 1*

*Isolation and sequence analysis of a potato  $\alpha$ -glucosidase clone.*

15 An Arabidopsis expressed sequence tag (EST) (European Molecular Biology Laboratory (EMBL) accession number; t04464.em\_est) with sequence similarity to the human lysosomal  $\alpha$ -glucosidase gene (Hoefsloot et al., 1988) was used as a probe to obtain the corresponding potato  
20 cDNA (pMAL1). The EST was generated in an Arabidopsis expressed sequence project and can be accessed as described in Newman et al., 1994. A potato cDNA (cultivar Record) library constructed in Zap II (Stratagene) from tuberising stolon tip mRNA (Taylor et al., 1992) was  
25 screened by standard techniques using this EST as a probe. Following in vivo excision and sub-cloning, DNA sequence of the pMAL1 clone was obtained for both strands using cycle sequencing (DyeDeoxy Terminator kit, Perkin Elmer) and a 373 automated DNA sequencer (Applied  
30 Biosystems). DNA sequence analysis was carried out using software available on the SEQNET Computational Molecular Biology Facility at SERC Daresbury Laboratory UK. The size of the insert in the pMAL1 was 2992bp (Fig. 1). An open reading frame from the nucleotide in position 62 to

a termination codon at position 2819 was identified. The sequence around the putative ATG initiation codon (A at position 51) is AACCATGA and shares some similarity with the preferred context for higher eukaryotes (CACCATGG, Kozak, 1987). The polypeptide encoded by this ORF has a molecular weight of 105.4 kD and an isoelectric point of 5.75. The deduced amino acid sequence of the *pMAL1* open reading frame was compared with sequences in the EMBL database. The most significant matches were with members of family 31 of glucosyl transferases which includes  $\alpha$ -glucosidases from human (Hoefsloot et al., 1988) and *Candida tsukubaensis* (Kinsella et al., 1991). The most similar full length sequence was that of a human lysosomal  $\alpha$ -glucosidase (Hoefsloot et al., 1988, Fig 2). Overall the sequences share 50% similarity and 29% identity. The two sequences were less similar in the amino-terminal portion (41% similarity, 16% identity over the first 280 amino acids) however over the remaining portion of the sequence there is 53% similarity and 34% identity. Clusters of identical amino acids occur throughout this region. One of the most highly conserved regions is adjacent to the aspartic acid residue at amino acid 516 of the potato sequence. Regions highly similar to this are at the active site of rabbit isomaltase and sucrase (Hunziker et al 1986). The *pMAL1* ORF was also compared with the deduced amino acid sequences of ESTs from *Arabidopsis*, rice and *C.elegans* that show similarity to  $\alpha$ -glucosidases. The *pMAL1* ORF shared 77%, 76% and 58% identity, respectively with these sequences (not shown).

#### Example 2

Complementation of a yeast  $\alpha$ -glucosidase mutant.

To confirm that the *pMAL1* clone did encode a functional  $\alpha$ -glucosidase gene, its ability to complement an  $\alpha$ -glucosidase-negative mutant strain of *Saccharomyces cerevisiae* was investigated. Such a mutant strain of *Saccharomyces cerevisiae* designated ABYSMAL81 (Kopetzki et al., 1989) has the genotype *ura3-52mal1S-<sup>+</sup>pralprb1prclcps1lys*. This mutant was used in transformation studies using standard yeast culture and manipulation methods as described by Rose et al., 1990. The open reading frame encoding the potato  $\alpha$ -glucosidase was cloned into the *Hind* III site of the yeast expression vector *pYES2* (Invitrogen) to generate the construct *pMAL1YES2* using standard techniques. The coding region of the cDNA clone was amplified using the Expand High Fidelity PCR system (Boehringer Mannheim) and the sequence of the PCR product was verified. PCR primers were designed to incorporate *Hind* III sites at the 5' and 3' termini of the fragment and the initiation codon was preceded by an AT rich sequence (TTAAA) in order to enhance efficient translation initiation (Romanos et al., 1995). The sequence of the primers used for building the yeast expression construct containing the amplified potato  $\alpha$ -glucosidase sequence were:

5' primer: cgaagcttaaaatgagagctccactactc  
3' primer: cccaagcttgaatcgaccaatcatc

The lithium acetate transformation method (Rose et al., 1990) was used to introduce the plasmids *pYES2* and *pMAL1YES2* independently into the yeast strain ABYSMAL81. Transformants were selected for uracil auxotrophy by standard procedures. Expression of the *MAL1* sequence in the *pMAL1YES2* construct was under the control of the *GAL1* portion of the *GAL1/GAL10* promoter which is inactive in



the absence of galactose, induced a 1000 fold in its presence and is subject to glucose repression (Johnston, 1987). Growth of pMAL1YES2 transformants was observed in the presence of 0.1% galactose and 2% maltose at a rate similar to that of the non-transformed mutant and vector transformed mutant growing on 2% glucose as the carbon source (Fig. 4). It was confirmed that the non-transformed mutant and vector transformed mutants were incapable of growth on this maltose/galactose medium and the transformant required the addition of galactose for growth. Presumably the level of galactose in the medium (0.1%) was too low to support growth of the mutant but was sufficient to induce expression from the GAL1 promoter.

$\alpha$ -Glucosidase activity in crude yeast extracts was determined in the transformed and non-transformed mutant strains. Overnight cultures of yeast (50 ml) were harvested by centrifugation (2000g, 5 min) and the cell pellet resuspended in 0.5 ml of 10 mM sodium phosphate buffer, pH 6.5. Cells were broken by sonication using a Misonix Ultrasonic Cell Disruptor (Misonix Inc) and cell debris was then sedimented by centrifugation (16000g, 20 min). Small molecules were removed from the supernatant by gel filtration using a PD-10 Sephadex G-25 M column (Pharmacia).  $\alpha$ -Glucosidase activity was determined using a range of maltodextrins, amylopectin and boiled soluble starch as substrates. The reaction mixture (100 L) contained 50mM maltodextrin or 2% (w/v) amylopectin or boiled soluble potato starch substrate in sodium phosphate buffer pH 6.5, and 50  $\mu$ L of crude extract and was incubated for 1 hour at 30°C. The reaction was terminated by boiling for 5 minutes and the amount of glucose released determined by the reduction of NAD<sup>+</sup> in

the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase in the presence of ATP and NAD<sup>+</sup> in a microtiter plate assay (Viola and Davies, 1992). Significant activity was detected following transformation of the ABYSMAL81 mutant with pMAL1YES2 and galactose induction. No activity could be detected in the non-transformed or pYES2 transformed ABYSMAL81 strain (Table 1). The optimum pH for  $\alpha$ -glucosidase activity was 6.5 (Fig.5).

The rate of hydrolysis of a range of maltodextrins, amylopectin, and boiled starch was also investigated (Table 1). The greatest activity was observed when maltotetraose was the substrate (151% of the maltose rate). Very little activity (5% of the maltose rate) was detected when amylopectin or boiled soluble starch were used as substrates although this was significantly greater than in the non-transformed mutant.

Table 1.  $\alpha$ -Glucosidase activity in crude extracts of ABYSMAL81 transformed with pMAL1YES2. The 100% maltose rate was 3.25 nmoles glucose/ g protein / hr.

5	<b>Substrate</b>	<b><math>\alpha</math>-glucosidase activity. (percentage of maltose rate)</b>
	maltose	100
	maltotriose	142
10	maltotetraose	151
	maltopentaose	117
	maltohexaose	68
	maltoheptaose	50
	amylopectin	5
15	boiled starch	5

### Example 3

#### Transformation studies.

Preliminary transformation studies were carried out using constructs containing the potato MAL1 sequence. Two constructs were built as described below. An antisense construct containing the entire MAL1 coding sequence in reverse orientation under the control of two copies of the constitutive CaMV 35S promoter and containing the nos terminator was cloned into the plant transformation vector pBIN19 (Bevan, 1984). This vector contained a kanamycin selection marker. A sense construct was built as above except the entire MAL1 coding region was cloned in the sense orientation. 59 independent (determined by Southern analysis (Sambrook et al., 1989)) transgenic potato antisense lines and 45 sense lines were generated in the potato cultivar Desiree following standard transformation protocols (Kumar, 1995).

## Example 4

 *$\alpha$ -Glucosidase activity measurements.*

*$\alpha$ -Glucosidase activity measurements were carried out (Cochrane et al., 1991) on tuber and leaf samples from the MAL1 antisense plants in Example 3. In tubers from these transgenic lines, a small (20%) but consistent decrease in activity was detected on either a protein or fresh weight basis in three lines. In leaf samples from the antisense lines a significant decrease in activity was measured, in some lines the activity was approximately 50% that observed in vector-only transformed leaves. Other lines showed a decrease in activity of up to 50%.*

## 15 Example 5

*Tuber sugar content.*

*Glucose, fructose and sucrose were assayed by high pH anion exchange chromatography with pulsed amperometric detection using a CarboPac PA-100 column following the manufacturer's protocol (Dionex) in samples of tissue from "first generation" transgenic tubers (tubers stored for three months at 4°C) (the antisense plants from Example 3). Compared with transgenic plants transformed with only the vector (pBIN19), a decrease in sucrose content was measured in some transgenic MAL1 antisense lines (table 2 below). In the line that exhibited the strongest effect, sucrose levels were approximately 30 to 60% of the control values.*

30 Table 2. Levels of glucose, fructose and sucrose in stored potato tubers from MAL1 antisense plants compared with those from vector-only transformed plants.

(Data from selected lines to show range of values

obtained in antisense plants, values for antisense plants mean of 5 replicates from different tubers of the same transgenic line or mean of 8 replicates for the vector-only transformed control.)

5

Transgenic line	glucose mg glu/gfr.wt.	fructose mg fru/gfr.wt.	sucrose mg glu/gfr.wt.
2M1	0.05	0.05	1.08
3M4	0.09	0.08	1.74
11E	0.19	0.28	0.84
12C	0.05	0.08	1.17
12D	0.10	0.13	1.10
control	0.08	0.11	1.89

10

15

#### Example 6

*Leaf starch turnover, maltose content and  $\alpha$ -glucosidase activity.*

20

25

30

The rate of starch turnover in fully expanded leaves from selected lines of antisense *MAL1* plants (from Example 3) was measured. Plants were grown in a growth cabinet at saturating light intensities with an 18 hour light period and a six hour dark period. Starch was extracted from leaves (Leidreiter et al. 1995) at the end of the light period and after four hours of darkness. The ratio of starch content in the light and dark was found to be consistently higher in the vector-only transformed lines (mean value 3.3) compared with the *MAL1* antisense lines (lowest values 2.4). The maltose content after 4 hours of darkness was also measured in leaf samples. Leaves from the *MAL1* antisense lines had up to twice the maltose content of those from vector-only transformed control lines. The results are shown in Table 3.

Table 3.

Starch content,  $\alpha$ -glucosidase activity and maltose levels in leaves from *MAL1* antisense plants and vector-only transformed plants (control) after 18 hours light treatment (L) and 4 hours dark treatment (D). Data represent mean values of 5 samples.

10	Transgenic line	$\alpha$ -glucosidase activity (vector value = 100%)	maltose	starch content	ratio(L/D)	
					mM glu/gfr.wt light	dark
	control	100	100	46.4	14.4	3.20
15	3M4	63	200	43.4	18.4	2.36
	11E	54	204	50.2	19.2	2.60
	14A	76	143	55.7	24.3	2.29

#### 20 Example 7

##### Targetted expression of the *MAL1* gene.

A construct has been designed in which the expression of the *MAL1* gene is under the control of the tuber-specific GBSSI promoter (Visser et al., 1989). The construct also contains the GBSSI targetting sequence so that the *MAL1* gene product was targetted to the amyloplast. Plants have been transformed with this construct and the *MAL1* gene product was expressed preferentially in the amyloplast.

30

##### Summary of the Transformation Studies.

Approximately 50 transgenic lines containing the *MAL1* sequence in sense and antisense orientation have been generated. In leaf samples of the antisense lines,

5  $\alpha$ -glucosidase activity was reduced by up to 50% compared with the vector control values. In tubers, few lines exhibited a consistent decrease in maltase activity and there was considerable tuber to tuber variability. Two  
10 consistent effects have been noticed however. Firstly, starch turnover rate (as determined by the ratio of light to dark starch content) was significantly lower in leaves from some MAL1 antisense lines. Maltose levels in leaves from these antisense plants were higher (up to double that routinely measured in leaf samples from control  
15 plants). Secondly, in stored tubers from the antisense lines, the sucrose level was consistently lower than in control tubers, in some lines the sucrose content was only 30 to 60% of that observed in the control tubers.

#### References.

20 Abdel-Akher, M., Hamilton, J.K., Montgomery, R. and Smith (1952) A new procedure for the determination of the fine structure of polysaccharides. J. Am. Chem. Soc. 74, 4970-4971.

25 Beck, E. and Ziegler, P. (1989) Biosynthesis and degradation of starch in higher plants. In W.R. Briggs, R.L. Jones and V. Walbot eds Ann Rev. Plant Phys and Mol. Biol. Vol 40 Annual Reviews, Palo Alto, CA, pp95-117.

30 Bevan, M.W. (1984) Binary Agrobacterium vectors for plant transformation. Nuc. Acids Res. 12, 8711-8721.

Chiba, S. (1988). Plant  $\alpha$ -glucosidases. In Amylase Research Society of Japan ed. Handbook of amylases and related enzymes. Permagon Press. New York. pp109-112.



Cochrane , M.P., Duffus, C.M., Allison, M.J. and Mackay, G.R. (1991). Measurement of amylolytic activity in potato tubers. *Potato Res.* 34, 325-332.

5 Coruzzi, G., Broglie, R., Edwards, C., and Chua, N-H, (1984). Tissue-specific and light regulated expression of a pea nuclear genome encoding the small subunit of ribulose-1,5-biphosphate carboxylase. *EMBO J.* 3, 1671-1679.

10

Draper, J., Scott, R.J., Armitage, P. and Walden, R. (1988) *Plant genetic transformation and gene expression: a laboratory manual*. Blackwell Scientific Publications, London.

15

Hoefsloot, L.H., Hoogeveen-Westerveld, M., Kroos, M.A., van Beeumen, J., Reuser, A.J.J. and Oostra, B.A. (1988) Primary structure and processing of lysosomal alpha-glucosidase: homology with the intestinal sucrase-isomaltase complex. *EMBO J.* 7, 1697-1704.

20

Hunziker, W., Spiess, M., Semenza, G. and Lodish, H.F. (1986) The sucrase-isomaltase complex: primary structure, membrane orientation and evolution of a stalked intrinsic brush border protein. *Cell*, 46, 227-234.

25

Johnston, M. and Davis, R.W. (1984) Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 4, 1440-1448.

30

Kim, SY., May, GD., and Park, WD., (1994). Nuclear-protein factors binding to a class-one patatin promoter region are tuber specific and sucrose inducible. *Plant. Mol. Biol.* 26, 603-615.

5 Kinsella, T., Hogan, S., Larkin, A. and Cantwell, B.A.  
(1991) Primary structure and processing of the *Candida*  
*tsukubaensis*  $\alpha$ -glucosidase. Homology with the rabbit  
intestinal sucrase-isomaltase complex and human lysosomal  
 $\alpha$ -glucosidase. Eur. J. Biochem. 202, 657-664.

10 Kopetzki, E., Buckel, P. and Schumacher, G. (1989)  
Cloning and characterization of baker's yeast alpha-  
glucosidase: over-expression in a yeast strain devoid of  
vacuolar proteinases. Yeast 5, 11-24.

15 Kozak, M. (1986) Point mutations define a sequence  
flanking the AUG initiator codon that modulates  
translation by eukaryotic ribosomes. Cell 44, 283-292.

20 Kumar, A. (1995) *Agrobacterium*-mediated transformation in  
potato genotypes. In *Agrobacterium protocol* (Gartland,  
K. and Davey, M.R., eds), Towata, NJ: Humana Press, pp.  
77-92. Leidreiter, K., Heineke, D., Heldt, H.W., Muller-  
Rober, B., Sonnewald, U. and Willmitzer, L. (1995) Leaf-  
specific antisense inhibition of starch biosynthesis in  
transgenic potato plants leads to an increase in  
photoassimilate export from source leaves during the  
light period. Plant Cell Physiol. 36, 615-624.

25 Lincoln, JE., Cordes, E., Read, E., and Fischer, RL.,  
(1987), Regulation of gene expression by ethylene during  
*Lycopersicon esculentum* (Tomato) fruit development. Proc.  
Natl. Acad. Sci. USA 84, 2793-2797.

30 Newman, T., de Bruijn, F.J., Green, P., Keegstra, K.,  
Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N.,  
Somerville, S., Thomashow, M., Retzel, E. and Somerville,  
C. (1994) Genes galore: a summary of methods for

accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* 106, 1241-1255.

5 Romanos, M. A., Scorer, C.A. and Clare, J.J. 1995 In: DNA Cloning 2 Expression Systems. Eds Glover D.M. and Hames, B.D. Oxford University Press, Oxford.

10 Rose, M.D., Winston, F. and Hieter, P. (1990) In: Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

15 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schwimmer, S. (1945) The role of maltase in the enzymolysis of raw starch. *J. Biol. Chem.* 161, 219-234.

20 Stamberg, O.E. and Bailey, C.H. (1939). Studies on wheat starch. II. The action of amylases on raw wheat starches. *Cereal Chem.* 16, 319-330.

25 Sun, Z., Duke, S.H. and Henson, C.A. (1995) The role of pea chloroplast alpha-glucosidase in transitory starch degradation. *Plant Physiol.* 108, 211-217.

30 Sun, Z. and Henson C.A. (1990) Degradation of native starch granules by barley alpha-glucosidases. *Plant Physiol.* 94, 320-327.

Taylor, M.A., Mad Arif, S.A., Kumar, A., Davies, H.V., Scobie, L.A., Pearce, S.R. and Flavell, A.J. (1992) Expression and sequence analysis of cDNAs induced during

the early stages of tuberisation in different organs of the potato plant (*Solanum tuberosum* L.). Plant Mol. Biol. 20, 641-651.

- 5 Visser, R.G.F., Hergersberg, M., Van der Leij, F.R., Jacobsen, E., Witholt, B., Feenstra, W.J. (1989). Molecular cloning and partial analysis of the gene for granule-bound starch synthase from a wildtype and an amylose-free potato (*Solanum tuberosum* L.). Plant Sci., 10 64, 185-192.

Viola, R. and Davies, H.V. (1992) A microplate reader assay for rapid enzymatic quantification of sugars in potato tubers. Potato Research 35, 55-58.

- 15 Wolffrom, M.L. and Thompson, A. (1956) Occurrence of the (1-3)-linkage in starches. J. Am. Chem. Soc. 78, 4116-4117.

- 20 Yamasaki, Y. and Konno, H. (1985) Two forms of  $\alpha$ -glucosidase from soybean callus. Agric. Biol. Chem. 51, 3239-3244.

- 25 Yamasaki, Y. and Suzuki, Y. (1980) Two forms of  $\alpha$ -glucosidase from sugar beet seeds. Planta 148, 354-361.

- 30 Yoshikawa, K., Yamamoto, K. and Shigetaka, O. 1994. Classification of some  $\alpha$ -glucosidases and  $\alpha$ -xylosidases on the basis of substrate specificity. Biosci. Biotech., Biochem. 58, 1392-1398.

**CLAIMS**

1. Recombinant or isolated nucleic acid encoding an  $\alpha$ -glucosidase enzyme, preferably a plant  $\alpha$ -glucosidase enzyme.

5

2. Recombinant or isolated nucleic acid as claimed in claim 1 which is obtainable from potato.

10

3. Recombinant or isolated nucleic acid as claimed in claim 2 which is obtainable from the potato cultivar Record.

15

4. Recombinant or isolated nucleic acid, as claimed in any one of claims 1 to 3, wherein the enzyme has the activity of a 105.4 kD  $\alpha$ -glucosidase enzyme of potato.

20

5. Recombinant or isolated nucleic acid as claimed in any one of claims 1 to 4 encoding at least part of the amino acid sequence as shown in Figure 2.

6. Recombinant or isolated nucleic acid as claimed in any one of claims 1 to 5, wherein a coding sequence is operatively linked to a promoter.

25

7. Recombinant or isolated nucleic acid comprising a promoter which naturally drives expression of a nucleic acid sequence encoding a plant  $\alpha$ -glucosidase enzyme.

30

8. Recombinant or isolated nucleic acid as claimed in claim 7, wherein the coding nucleic acid sequence is as claimed in any one of claims 1 to 7.

9. Recombinant or isolated nucleic acid as claimed in any claim 6 to 8 which, when expressed, results in

enhanced starch and/or other carbohydrate breakdown in a plant or microbe.

5 10. Recombinant or isolated nucleic acid as claimed in any one of claims 6 to 9 including a signal sequence in translational fusion with the  $\alpha$ -glucosidase coding sequence

10 11. Antisense nucleic acid which comprises a transcribable strand of nucleic acid, which is complementary to at least part of the strand of nucleic acid which is transcribed from a nucleic acid sequence encoding a  $\alpha$ -glucosidase enzyme, preferably a plant  $\alpha$ -glucosidase enzyme.

15 12. Antisense nucleic acid as claimed in claim 11 wherein the sequence encoding the  $\alpha$ -glucosidase enzyme is obtainable from potato.

20 13. Antisense nucleic acid as claimed in claim 12, wherein the sequence encoding the  $\alpha$ -glucosidase enzyme is obtainable from the potato cultivar Record.

25 14. Antisense nucleic acid as claimed in claim 13, wherein the  $\alpha$ -glucosidase enzyme has the activity of a 105.4 kD  $\alpha$ -glucosidase enzyme of potato.

30 15. Antisense nucleic acid as claimed in any one of claims 11 to 14 which is complementary to at least part of the nucleic acid sequence encoding the amino acid sequence as shown in Figure 2.

16. Antisense nucleic acid as claimed in any one of claims 11 to 15 operatively linked to a promoter.

17. Nucleic acid capable of specifically disrupting the proper expression of an  $\alpha$ -glucosidase gene, preferably a plant  $\alpha$ -glucosidase gene.

5 18. Nucleic acid as claimed in claim 17, wherein the  $\alpha$ -glucosidase gene encodes a 105.4 kD  $\alpha$ -glucosidase enzyme of potato.

10 19. Nucleic acid as claimed in any one of claims 1 to 18 comprising a 5' transcription regulation sequence.

15 20. Nucleic acid as claimed in claim 19, wherein the 5' transcription regulation sequence is derived from the Cauliflower Mosaic Virus 35S gene.

21. Nucleic acid as claimed in any one of claims 1 to 20 which is recombinant and comprises part of a vector.

20 22. Nucleic acid as claimed in claim 21, wherein the vector is a cloning or an expression vector and comprises one or more selectable markers.

23. A host cell transfected or transformed with a vector as claimed in claim 21 or claim 22.

25 24. A plant or microbial cell comprising nucleic acid as claimed in any one of claims 1 to 10 or 19 to 23 (when dependent on any one of claims 1 to 10).

30 25. A plant or microbial cell comprising nucleic acid as claimed in any one of claims 11 to 18 or 19 to 23 (when dependent on any one of claims 11 to 18).

26. A plant, microbe, or part of a plant, at least some



of whose cells are as claimed in claim 24.

27. A plant, microbe, or part of a plant, at least some of whose cells are as claimed in claim 25.

5

28. Use of nucleic acid as claimed in any one of claims 1 to 22 to modify starch and/or other carbohydrate breakdown in plant or microbial material.

10

29. Use of nucleic acid as claimed in any one of claims 1 to 10 or 19 to 23 (when dependent on any one of claims 1 to 10) to obtain enhanced  $\alpha$ -glucosidase activity in plant or microbial material.

15

30. Use of nucleic acid as claimed in claim 29 to enhance flavour characteristics of plant tissue.

31. Use of a plant, or part of a plant, as claimed in claim 24 in a malting or brewing process.

20

32. Use of nucleic acid as claimed in any one of claims 11 to 18 or 19 to 23 (when dependent on any one of claims 11 to 18) to obtain reduced  $\alpha$ -glucosidase activity in plant or microbial material.

25

33. Use of nucleic acid as claimed in claim 32 to control pre-sprouting in cereals, sprout control in potato or loss of starch and/or dry weight in storage, cooking or processing of plant material.

30

34. A novel starch structure obtainable by expression of nucleic acid as claimed in any one of claims 1 to 22.

1 / 6

1	CGACGGCCAGTGAATTCCGGTCTTCTATGATATCTTTCCACATTGATCTCCGATTATAAC	60
61	CATGAGAGCTCCACTACTCCTATATCCACTCCTTCTCCTCCTTCTATTTGTTACCTCTGC	120
121	CTACTCCTGGAAGAAGGAGGAGTTTCGAAACTGCGACCAAACCCCATTTTGCAAAAGAGC	180
181	CCGTTCCTCGAAAACCTGGATCGTGCAATCTGCGGGTTGCCGATGTGTCCATCTCCGATGG	240
241	GGATCTTATAGCCAAACTTGTCCCCAAAGAAGAAAACCCAGAAAGTGAACAACCCAATAA	300
301	GCCTTTGGTTCTCACTCTTTCTGTGTACCAAGATGGTGTGATGAGGGTGAAAATTGATGA	360
361	AGATCAAAATCTGAATCCACCCAAGAAAAGATTTGAAGTTCCTGAGGTGATTGAGGAAGA	420
421	TTTCCTCAACACCAAGCTGTGGTTAACCAGAGTAAAAGAGGAGCAAATCGACGGAGTTTC	480
481	GAGTTTTTCTCTGTTTTTTACTTGTCTGATGGGTATGAAGGGGTGTTGAGACATGACCC	540
541	ATTTGAGGTTTTTGGCCAGAGAGAGTGGAAGTGGGAAGAGAGTGTTGTCCATAAACTCAA	600
601	TGGGTTGTTTTGATTTTGAACAGTTGAGGGAGAAGAAAGAAGGGGATGATTGGGAGGAGAA	660
661	GTTTAGGAGTCATACTGATACAAGGCCTTATGGTCCACAATCAATTAGTTTTGATGTATC	720
721	TTTTTATGGTGCAGATTTTGTATTATGGCATTCTGAACATGCTACTAGTTTTGCTTTGAA	780
781	ACCAACTAAGGGGCCCTAATGTAGAGGAATATTCCGAGCCTTATAGGTTATTTAATCTTGA	840
841	TGTGTTTGAGTATCTTCATGAGTCGCCTTTTGGGCTTTATGGTTCAATTCCTTTTCATGAT	900
901	TTACATGGGAAAGCCAGGGGTAGTTCGGGTTTTTCTGGTTGAATGCTGCGGAAATGCA	960
961	GATTGATGTATTGGGATCTGGTTGGAATTCAGATGAGTCTTCAAAGATAATGTTGCCCTC	1020
1021	GGACAAGCACAGGATTGATACTTTATGGATGAGTGAGTCTGGTGTAGTGGATACGTTCTT	1080
1081	TTTCATTGGTCCTGGGCCAAAAGATGTGGTTAGGCAGTATACTAGTGTAACGGGAAGGCC	1140
1141	ATCTATGCCACAGTTATTCGCAACTGCATACCATCAATGTAGATGGAATTATAGAGACGA	1200
1201	GGAAGATGTTTATAATGTTGATTCAAAATTTGATGAGCATGATATCCCTTATGATGTTTT	1260
1261	GTGGCTTGATATTGAGCACACAGATGGGAAGAAGTACTTTACTTGGGACAGGGTGTTATT	1320
1321	TCCTAACCCGGAAGAAATGCAGAAGAAGTTAGCTGCAAAGGGTAGACACATGGTTACCAT	1380
1381	TGTGGATCCTCATATCAAGAGGGATGAGTCTTACCATATAACCAAGGAGGCCTTAGAAAA	1440
1441	GGGATACTATGTTAAGGATGCTACTGGTAAGGATTATGATGGATGGTGCTGGCCTGGTTC	1500
1501	CTCATCATATACTGACTTGCTGAATCCCGAGATTAAGTCATGGTGGAGTGACAAATTTTC	1560
1561	ACTTGATAGCTATGTTGGCTCAACAAAGTATTTATACATCTGGAATGACATGAATGAACC	1620
1621	TTCCGTCTTCAATGGACCAGAGGTAACAATGCCAAGAGATGCTTTACATCATGGAGGAGT	1680
1681	AGAGCACAGGGAGTTGCACAATTCATATGGTTACTATTTCCATATGGGAACATCCGACGG	1740
1741	GCTTCTAAAGCGTGGAGATGGAAAAGATAGGCCTTTTGTGTTTGGCAAGGGCCTTCTTTC	1800
1801	CGGAAGTCAAAGATATGGAGCAATTTGGACTGGAGATAATACAGCAGAATGGGAGCACTT	1860
1861	GAGGGTTTCAGTCCCCATGGTGTAACTCTTAGCATCTCTGGAATAGTATTTTCTGGTGC	1920
1921	AGATGTTGGTGGATTTTGTGTAATCCTGACACTGAGTTGTTGGTTCGCTGGTATCAAGT	1980
1981	AGGTGCATATTATCCCTTTTTCCGGGGGCATGCACATCATGACACTAAAAGACGGGAACC	2040
2041	TTGGTTATTTGGAGAAAGAAATACACAATTGATGAGGGAAGCGATACATGTTTCGTTACAT	2100
2101	GTATCTTCTTATTTCTACACTCTATTTAGAGAAGCAAATTCAAGTGGTACTCCAGTTGC	2160
2161	TCGCCCCTTTGGATGGAGTTCCCTGGAGACGAAAAATCTTTTAGCAATGATGAGGCTTT	2220
2221	CATGGTTGGGAATGGTCTTCTAGTGCAAGGAGTTTATACAGAGAAACCAAAACATGTTTC	2280
2281	TGTCTATCTACCAGGGGAGGAATCCTGGTATGATTTAAGAAGTGCATCTGCATACAACGG	2340
2341	AGGTCTACACACAAGTATGAGGTTTCAGAAGATAGTATTCCTTCTTTTCAAAGGGCCGG	2400
2401	AACTATCATACCAAGGAAAGATCGTTTACGTCCGAGCTCGACACAGATGGAAAATGATCC	2460
2461	TTATACTCTGGTTATAGCTCTTAATAGTTCCAAGGCAGCTGAAGGTGAGCTTTATATCGA	2520
2521	TGATGGGAAGAGCTATGAGTTCAAACAAGGTGCCTTCATTCTCAAATGGGAGGCTTATAT	2580
2581	CTTCCAAATGCAGCCCCGTCTACAGCTGGCAGTGACACATTTTCTTCCGAGTGCACTGT	2640
2641	AGAGAGGATAATCTTGTTAGGATTGTCTCCTGGAGCTAAAACAGCCCTTATTGAACCAGG	2700
2701	AAACAAGAAAGTTGAAATTGAGCTTGGGCCACTCTTCATTCAAGGAAATCGAGGATCTGT	2760
2761	TCCAACCATCCGCAAGCCTAATGTGCGTATTACAGATGATTGGTCGATTCAAATTTTGTA	2820
2821	AGAAGTTGGTAGTTATGACGAAGTCTTACATTTTCTCTTCCGCGTTTCATCATGTATTGG	2880
2881	GTTAGACCAGCTAATAGGTAAATCTTTACTAGAAGAATTAAACATTGATGAAGATTGTTA	2940
2941	TACAGGGATAGTTTGAAGCGGCCGCGAATTCGAGCTCGGTACCCGGGGATCC	2992

FIG. 1.

2 / 6

1 MRAPLLLYPL LLLLLFV TSA YSWKKEEFRN CDQTPFCKRA RSRKPGSCNL  
51 RVADVSI SDG DLI AKLVPKE ENPESEQPNK PLVLTLSVYQ DGVMRVKIDE  
101 DQNLNPPKKR FEVPEVIEED FLNTKLWLTR VKEEQIDGVS SFSSVFYLS D  
151 GYEGVLRHDP FEVFARESGS GKRVL SINSN GLFD FEQLRE KKEGDDWEEK  
201 FRSHTDTRPY GPQSI SFDVS FYGADFVYGI PEHATSFALK PTKGPNVEEY  
251 SEPYRLF NLD VFEYLHESPF GLYGSIPFMI SHGKARGSSG FFWLNAAEMQ  
301 IDVLGSGWNS DESSKIMLPS DKHRIDTLWM SESGVVD TFF FIGPGPKDVV  
351 RQYTSVTGRP SMPQLFATAY HQCRWNYRDE EDVYNVDSKF DEHDIPYDVL  
401 WLDIEHTDGK KYFTWDRVLF PNPEEMQKKL AAKGRHMTI VDPHIKRDES  
451 YHIPKEALEK GYYVKDATGK DYDGWCWPGS SSYTDLLNPE IKS WWS DKFS  
501 LDSYVGSTKY LYIWNDMNEP SVFNGPEVTM PRDALHHGGV EHRELHNSYG  
551 YYFHMGTSDG LLKRGDGKDR PFVLARAFFA GSQRYGAIWT GDNTAEWEHL  
601 RVSVP MVLT L SISGIVFSGA DVGGFFGNPD TELLVRWYQV GAYYPFFRGH  
651 AHHDTKRREP WLFGERNTQL MREAIHVRYM YLPYFYTLFR EANS SGTPVA  
701 RPLWMEFP GD EKSFSNDEAF MVGNLLVQG VYTEKPKHVS VYLPGEESWY  
751 DLRSASAYNG GH THKYEVSE DSIPSFQ RAG TIIPRKDRLR RSSTQMENDP  
801 YTLVIALNSS KAAEGELYID DGKSYEFKQG AFILKWEAYI FQM QPRLQLA  
851 VTHFPSECTV ERIILLGLSP GAKTALIEPG NKKVEIELGP LFIQGNRGSV  
901 PTIRKPNVRI TDDWSIQIL

FIG. 2.

3 / 6

pot	1	.....MRAPLLLYPLLLLLLFVT...SAYSWKKE	25
hum	1	MGVRHPPCSHRL LAVCALVSLATAALLGHILLHDFLLVPREL SGSSPVLE	50
pot	27	EFNRCDQTPFCKRARSRKPGSCNLRVADVSI SDGD LIAKLVPKEENPESE	76
hum	51	ETHPAHQQGASRPGPRDAQAHFGRPRAVPTQCDVPPNSRFD CAPDKAITQ	100
pot	77	QPNKPLVLTLSVYQDGVMRVKIDEDQNLNP...PKKRFEVPEVIEEDFLN	123
hum	101	EQCEARGCCYIPAKQGLQGAQMGPWCFFPPSYPSYKLENLSSSEMGYTA	150
pot	124	TKLWLTR.....VKEEQIDGVSSFSV FYLS DGYEGLRHD.PFEVFAR	166
hum	151	TLTRTTPTFFPKDILTLRLDVMETENRLHFTIKDPANRRYEVPLETPRV	200
pot	167	ESGSGKRVLSIN.SNGLFDFEQLREKKEGDDWEEKFRSHTDTRP..YGPO	213
hum	201	HSRAPSPLYSVEFSEEPFGVIVHR.....QLDGRVLLNTTVAPLFFADQ	244
pot	214	SISFDVSFYGADFVYGIPEHATSFALKPTRKGNVEEYSEPYRLFNLDVFE	263
hum	245	FLQLSTSL.PSQYITGLAEHLSPLMLSTS.....WTRITLWNRDL..	283
pot	264	YLHESPFGLYGSIPFMISHGKARGSSGFFWLNAEMQIDVLGSGWNSDES	313
hum	284	.APTPGANLYGSHPFYLALEDGGSAGVFLNLSNAMDVVLQPSALS...	329
pot	314	SKIMLP SDKHRIDTLWMSESGVVD TFFF IGPGPKDVVRQYTSVTGRPSMP	363
hum	330	.....WRSTGGILDVYIFLGPEPKSVVQQYLDVVGYPFMP	364
pot	364	QLFATAYHQCRWNYRDEEDVYNVDSKFDEHDI PYDVLWLD IEHTDGKKYF	413
hum	365	PYWGLGFHL CRWGYSS TAITRQVVENMTRAHFPLDVQWNDLDYMDSR RDF	414
pot	414	TWDRVLF PNPEEMQKKLAAGRHMTIVDPHIK...RDESYHIPKEALEK	460
hum	415	TFNKDGF RDFFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYR PYDEGLRR	464
pot	461	GYVVKDATGKDYDGWCWPGSSSYTDLLNPEIKSWWSDKFSLDSYVGSTKY	510
hum	465	GVFITNETGOPLIGKVWPGSTAFPDFTNPTALAWWEDM..VAEFHDQVPF	512
pot	511	LYIWNDMNEPSVF.NGPEVTMPRDALHHGG.....	539
hum	513	DGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVG GTLOAATICASSH	562

FIG. 3.

SUBSTITUTE SHEET (RULE 26)

4 / 6

pot	540	...VEHRELHNSYGYFHMGTSDGLLKRGDGKDRPFVLARAFFAGSORYG	586
hum	563	QFLSTHYNLHNLGLTEAIAASHRALVKARGT...RPFVISRSTFAGHGRYA	610
pot	587	AIWTGDNTAEWEHLRVSVPMVLTLSISGIVFSGADVGGFFGNPDTELLVR	636
hum	611	GHWTGDVWSSWEQLASSVPEILOFNLLGVPLVGADVCGFLGNTSEELCVR	660
pot	637	WYQVGAYYPFFRGHAHDTKRREPWLFGERNTOQMREAIHVRYMYLPYFY	686
hum	661	WTQLGAFYPPMRNHNLSLPOEPYSFSEPAQQAMRKALTRYALLPHLY	710
pot	687	TLFREANSSGTPVARPLWMEFPGDEKSFSNDEAFMVGNGLLVQGVYTEKP	736
hum	721	TLFHQAHVAGETVARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLOAGK	760
pot	737	KHVSVYLPGEESWYDL.....RSASAYNGGHTHKYEY	768
hum	761	AEVTGYFP.LGTWYDLOTVP IEALGSLPPPPAAPREPAIHSEGQWVTLPA	809
pot	769	SEDSIPSFORAGTIIPRKDRLRRSSTOMENDPYTLVIALNSSKAAEGELY	818
hum	810	PLDTINVHLRAGYIIPLOGP.GLTTTESRQOPMALAVALTKGGEARGELF	858
pot	819	IDDGKSIEFKQGAFILKWEAYIFQMOPRLQLAVTHFPSECTVERIILLGL	868
hum	859	WDDGESLE.....VLERGAY.....TQVIFLAR	881
pot	869	SPGAKTALIEPGNKKVEIELGPLFIQGNRGSVPTIRKPNVRITDDWSIQI	918
hum	882	NNTIVNELVRVTSEGAGLOLOKQVTVLG....VATAPO.....QV	916
pot	919	L*	
hum	917	LSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC.....	952

FIG. 3 contd.

5 / 6

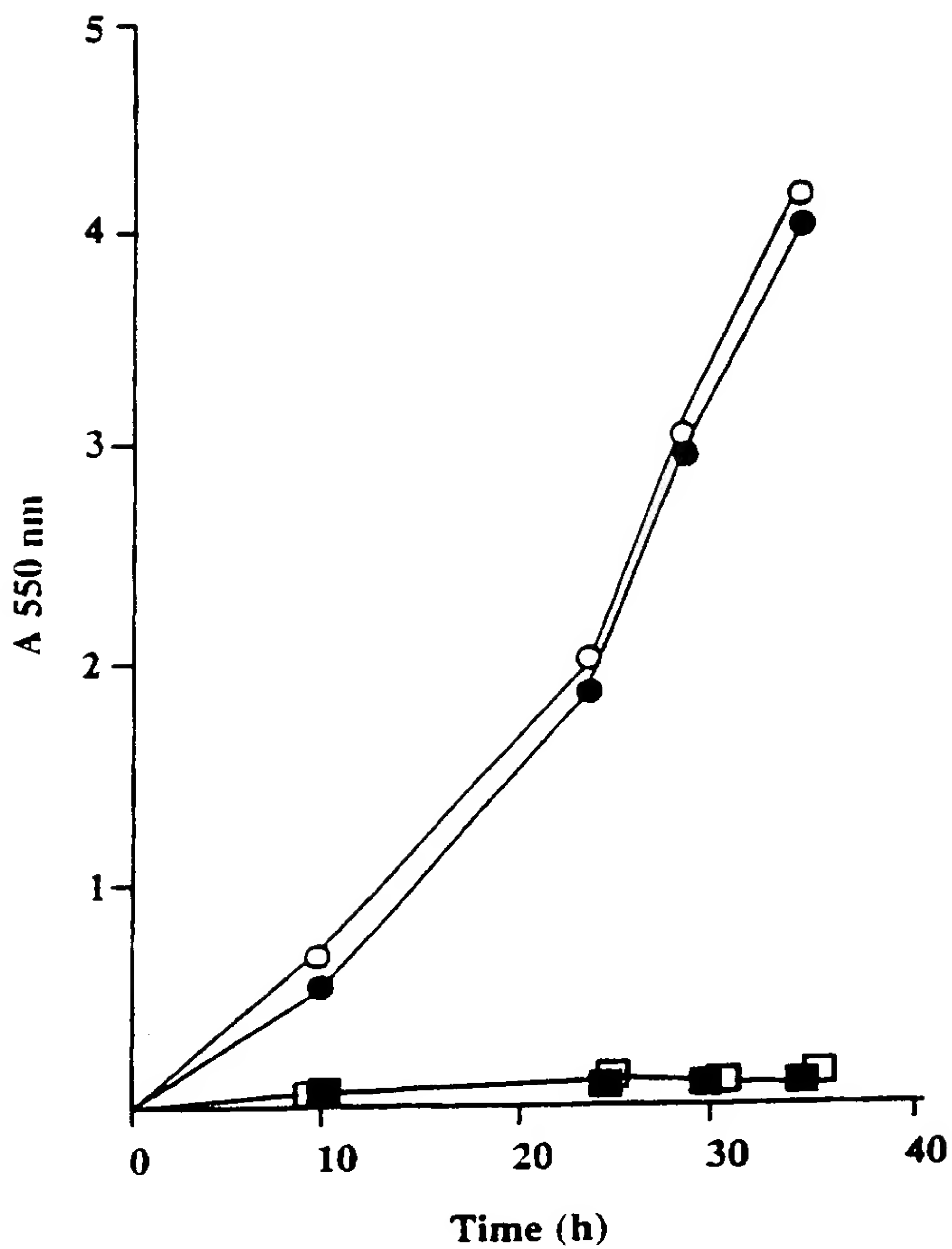


FIG. 4.

6 / 6

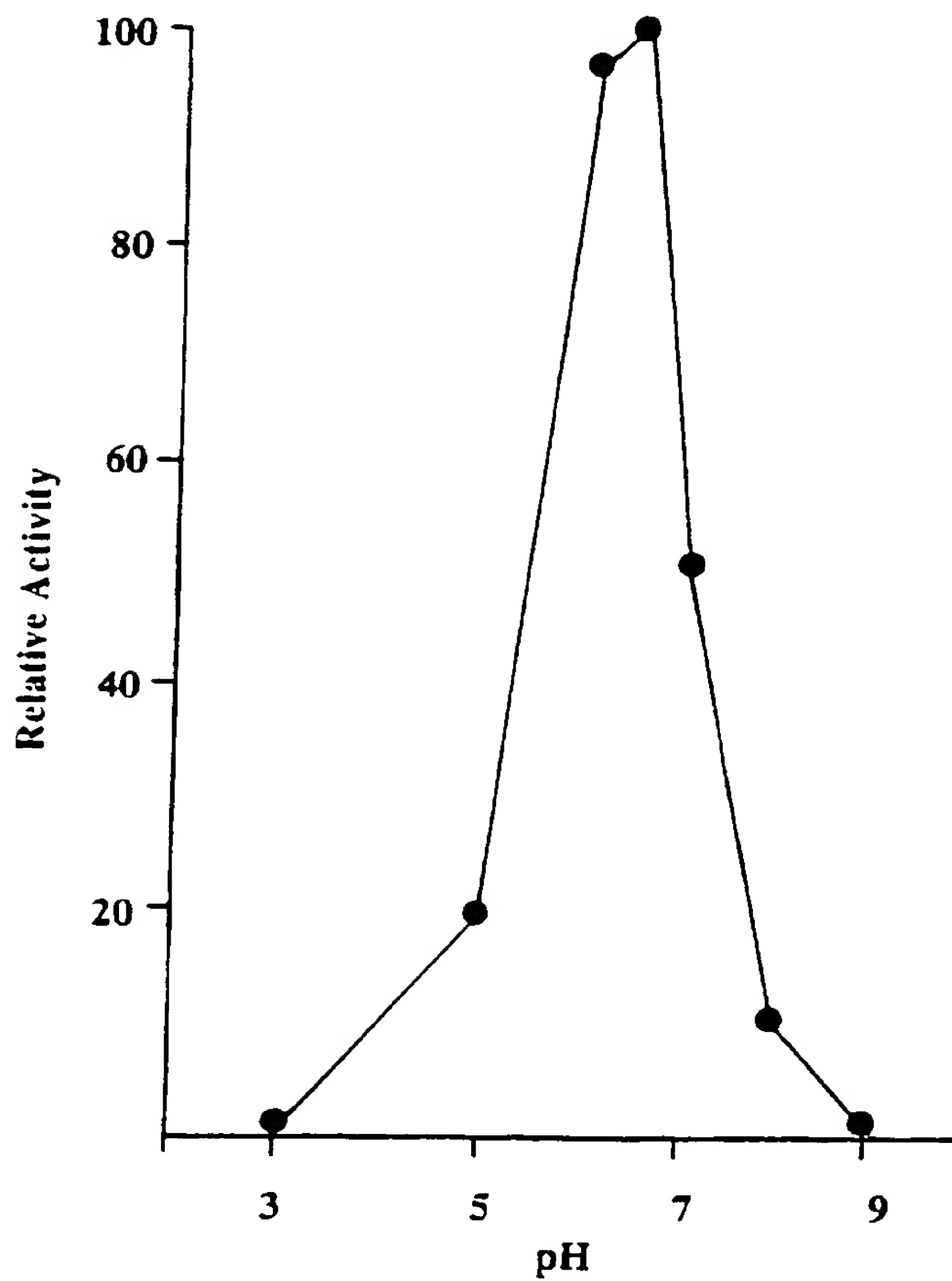


FIG. 5.



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 96/03239

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/82 C12N15/55 C12N1/21 C12N1/18 C12N5/10  
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 42 13 444 A (INST GENBIOLOGISCHE FORSCHUNG) 28 October 1993  see column 3, line 9 - line 41; claims 1-3 ---	1,6,11, 16,17, 19, 21-28, 32-34
X	EMBL SEQUENCE DATABASE, REL. 44, 17-AUG-1995, ACCESSION NO. U22450, XP002029013 TIBBOT, B.K., ET AL.: "Hordeum vulgare alpha-glucosidase mRNA, complete cds." see sequence ---	1,5,9, 11,15, 17,21-25
X	GB 2 247 238 A (GUINNESS SON & CO LTD A) 26 February 1992  see the whole document ---	1,5,6, 9-11,19, 21-29

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

8 April 1997

Date of mailing of the international search report

14. 04. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Maddox, A

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/03239

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 260 404 A (HEINEKEN TECHNISCHE BEHEER BV) 23 March 1988  see the whole document ---	1,5,6, 9-11,19, 21-29
X	THE EMBO JOURNAL, vol. 7, no. 6, 1988, pages 1697-1704, XP002029014 HOEFSLOOT, L.H., ET AL.: "Primary structure and processing of lysosomal alpha-glucosidase; homology with the intestinal sucrase-isomaltase complex" cited in the application see the whole document ---	1
X	EMBL SEQUENCE DATABASE, REL.36, 30-AUG-1993, ACCESSION NO. T04464, XP002029015 NEWMAN, T., ET AL.: "511 Arabidopsis thaliana cDNA clone 35C12T7" cited in the application see sequence ---	5
X	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, vol. 15D, 1991, page 24 XP002029016 CLAUSEN, I.G., ET AL.: "Suppression by antisense mRNA of synthesis from highly expressed genes in Aspergillus oryzae and Aspergillus niger" see abstract CD305 ---	11
X	EP 0 479 359 A (GIST BROCADES NV ;MOGEN INT (NL)) 8 April 1992 see page 4, line 18; claim 10; example 12 see page 7, line 42 - line 49 ---	1,6,9, 24-30,34
A	WO 90 12876 A (DANSKE SPRITFABRIKKER) 1 November 1990 see page 104 - page 107 ---	11
A	JAPANESE JOURNAL OF BREEDING, vol. 67, 1992, pages 147-154, XP002029017 TSUTSUMI, N., ET AL.: "Suppression of alpha-amylase gene expression by antisense oligonucleotide in cultured barley aleurone layers" see the whole document ---	11

-/--

## INTERNATIONAL SEARCH REPORT

Patent Application No  
PCT/GB 96/03239

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AUST. SOC. BIOCHEM. MOL. BIOL., (1994) 26, COL-5-4, 1994, XP000617525 VICKERS, J.E., ET AL.: "Thermostable alpha-amylase cloned for genetic transformation of barley" see abstract. ---	31
A	PLANT PHYSIOLOGY, vol. 108, May 1995, pages 211-217, XP002029018 SUN, Z., ET AL.: "The role of pea chloroplast alpha-glucosidase in transitory starch degradation" cited in the application see the whole document -----	1-34

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/03239

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4213444 A	28-10-93	NONE	
GB 2247238 A	26-02-92	NONE	
EP 0260404 A	23-03-88	EP 0257115 A	02-03-88
		AU 621810 B	26-03-92
		AU 7685087 A	16-06-88
		IE 69658 B	02-10-96
		AT 123304 T	15-06-95
		DE 3751326 D	06-07-95
		DE 3751326 T	11-01-96
		ES 2003314 T	01-10-96
		JP 63133984 A	06-06-88
		US 5100794 A	31-03-92
		US 5151354 A	29-09-92
		ZA 8706134 A	29-02-88
EP 0479359 A	08-04-92	AU 656920 B	23-02-95
		AU 8651491 A	15-04-92
		CA 2072656 A	14-03-92
		JP 5502591 T	13-05-93
		WO 9205259 A	02-04-92
		NZ 239789 A	27-06-94
WO 9012876 A	01-11-90	AU 638248 B	24-06-93
		AU 5531890 A	16-11-90
		CA 2053230 A	25-10-90
		DE 69008172 D	19-05-94
		DE 69008172 T	20-10-94
		EP 0470145 A	12-02-92
		ES 2053189 T	16-07-94
		IE 63067 B	22-03-95
		LV 10727 B	20-02-96
		US 5498832 A	12-03-96